stained with anti-Rap1 polyclonal antibody (sc-65) and goat antibody to rabbit immunoglobulin conjugated with Alexa 488 (Molecular Probes, Eugene, OR, USA). B-Raf was stained with anti-B-Raf monoclonal antibody (F-7) and chicken antibody to mouse immunoglobulin conjugated with Cy3 (Amersham Biosciences).

Detection of BRAF mutations

DNA was isolated from melanoma cell lines according to the standard methods. Exons 11 and 15 of BRAF cDNA were amplified by PCR using the following primer pairs (Davies *et al*., 2002): exon 11, sense 5′-TCCCTCTCSGGCATAAGG TAA-3′ and antisense 5′-CGAACAGTGAATATTTCCTTT GAT-3′; exon 15, sense 5′-TCATAATGCTTGCTCTGATA GGA-3′ and antisense 5′-GGCCAAAAATTTAATCAGTG GA-3′. The PCR products were subjected to direct DNA sequencing after purification.

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ORIGINAL ARTICLE

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Pilot phase II study of weekly chemotherapy with paclitaxel and carboplatin for refractory or relapsed small-cell lung cancer

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Abstract *Purpose*: The safety and efficacy of weekly chemotherapy with paclitaxel and carboplatin for the treatment of patients with refractory or relapsed small-cell lung cancer (SCLC) were evaluated. *Patients and methods*: Paclitaxel (100 mg/m^2) and carboplatin (with a target area under the concentration versus time curve of 2 mg min/ml using the Calvert formula) were administered to patients with previously- treated SCLC on days 1 and 8 at every 3–4 weeks. *Results*: A total of 29 patients (pts) [male/female, 26/3 pts; median age 62.7 years (43–74); performance status 0/1/2, 9/10/10 pts] were enrolled between March 2000 and June 2002. The mean number of cycles administered per pt was 3 (1–7). The overall response rate was 69% (95% confidence interval 52–86%), and 83% (15/18) in sensitive pts and 45% (5/11) in refractory pts ($P<0.01$). The overall median survival time was 29.6 weeks with a 1-year survival rate of 37% [34.1 weeks in sensitive pts and 23.1 weeks in refractory pts ($P=0.085$), 46.9 weeks in PS 0–1 and 16.3 weeks in PS 2 ($P<0.001$)]. The median time to progressive disease was 16.4 weeks [21.7 weeks in sensitive pts and 15.3 weeks in refractory pts ($P=0.32$)]. Hematologic toxicities observed included grade ≥3 neutropenia in 55%, grade ≥3 anemia in 36%, and grade ≥3 thrombocytopenia in 3%. Non-hematologic toxicities were mild except for grade 3 diarrhea in three pts and grade 3 pneumonitis in one pt. *Conclusion*: Weekly chemotherapy with paclitaxel and carboplatin was well-tolerated and gave a high-response rate in pts with refractory or relapsed small-cell lung cancer.

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Introduction

Small-cell lung cancer (SCLC) accounts for 15–20% of the total number of lung cancer patients. It grows more rapidly and shows a higher incidence of remote metastasis than non-small-cell lung cancer (NSCLC). It is apparently more sensitive to chemotherapy and radiotherapy than NSCLC, but is cured only in a small number of patients and recurs in a great majority of them. Recurrent SCLC is less responsive to chemotherapy, and the median survival time from recurrence to death is 2–3 months [3]. Chemotherapy has been reported to contribute to the improvement of symptoms and prolongation of the survival time in patients with recurrent SCLC [2, 6]. In general, first-line chemotherapy is conducted for sensitive disease (relapse ≥90 days after completion of first-line chemotherapy). For refractory disease (relapse during first-line chemotherapy or less than 90 days after completion of initial chemotherapy), however, salvage chemotherapy is undertaken due to the lack of a standard chemotherapy regimen. However, no standard chemotherapy has been established for recurrent SCLC [17].

In recent years, a number of institutions have undertaken weekly chemotherapy for lung cancer and reported the outcome [11, 14]. Weekly chemotherapy is being reported to be useful for recurrent SCLC as well [1, 4, 7, 10]. It is considered to be more suitable than the standard chemotherapy conducted every 3–4 weeks for recurrent cases with impaired bone marrow due to initial chemotherapy because it uses smaller doses of anti-cancer drugs in each administration cycle and it is possible to titrate their doses after starting the treatment depending on hemotoxicity and the patients' physical condition.

When used alone, paclitaxel was reported to produce good therapeutic results in patients with refractory SCLC with a response rate of 29% and a median survival time of 100 days [15]. When coadministered with carboplatin, paclitaxel showed even better results with a response rate of 73.5% and a median survival time of 31 weeks [5]. This report prompted us to conduct the present study to evaluate the efficacy and safety of weekly chemotherapy using carboplatin and paclitaxel in recurrent SCLC patients.

Patients and methods

Patient selection

All patients with histologically or cytologically confirmed SCLC with documented progression after chemotherapy were eligible for this phase II trial. Patients with either limited- or extensive-stage disease were allowed. The trial was initiated after a rest period of at least 4 weeks following previous chemotherapy (2 weeks in the case of radiotherapy). Patients were required to have recovered completely from prior therapy, with no ongoing toxicity greater than grade 1.

Other eligibility criteria included expected survival of 12 weeks, age $\leq$ 75 years, Eastern cooperative oncology group performance score of 0–2, measurable lesions, and adequate hematological function. Primary refractory disease was defined as relapse during first-line chemotherapy or less than 90 days after completing initial chemotherapy, and sensitive disease was defined as relapse $\geq$90 days after completion of first-line chemotherapy.

The ethical committee of the Tochigi cancer center approved the protocols. Written informed consent stating that the patient was aware of the investigational nature of this treatment regimen was obtained in every case.

Treatment

Paclitaxel was administered at a dose of 100 mg/m^2 intravenously during a 1-h infusion on days 1and 8 of the treatment cycle. Carboplatin was given at a dose designed to give an area under the curve (AUC) of 2 on days 1 and 8 with the use of the Calvert formula: 2 $\times$ (creatinine clearance + 25). Prior to each treatment, patients were given 50 mg diphenhydramine orally, and an H2 blocker intravenously along with 16 mg dexamethasone . Intrvenously administered antiemetics, 3 mg graniston, were used. The length of each chemotherapy cycle was 21 days. Patients who experienced grade 4 leukopenia or neutropenia that lasted for three days or more , or who experienced grade 4 thrombocytopenia, reversible grade 2 neurotoxicity, or liver dysfunction, received reduced doses of both paclitaxel and carboplatin (paclitaxel 80 mg/m^2, carboplatin AUC1.5) for the next cycle. If non-hematologic toxicities of grade 3 or more occurred, treatment was stopped. Subsequent courses of chemotherapy were started after 3–4 weeks when the leukocyte count was 3,000/mm^3 or more, the neutrophil count 1,500/mm^3 or more, the platelet count 75,000/mm^3 or more, serum creatinine less than 1.5 mg/dl, GOT and GPT less than twice the upper limit of the normal range, and neurotoxicity was grade 1 or less. If these variables did not return to adequate levels by the first day of the next course of chemotherapy, treatment was withheld until full recovery. If more than 6 weeks passed from the time of the last treatment before these criteria were satisfied, or if more than dose reduction were indicated, the patient was taken off the study at that time, but still included in the analysis.

Evaluation of response and toxicity

Pretreatment evaluation included medical history, physical examination, complete blood count, bone marrow examination, serum biochemical analyses, chest roentgenogram, electrocardiogram, and urinalysis. All patients underwent radionuclide bone scan, bone marrow aspiration or biopsy, magnetic resonance or computerized tomography (CT) of the brain, and CT of thorax and abdomen. Complete blood count, biochemical tests, serum electrolytes, urinalysis, and chest roentgenograms were obtained weekly during this phase II trial.

Response and toxicity were evaluated on the basis of tumor images obtained by CT and other techniques, laboratory data and subjective/objective symptoms before, during, and after administration of the study drugs and during the period from completion of treatment to final analysis. Measurable disease parameters were determined every 4 weeks by various means such as CT. Evaluation was made in compliance with response evaluation criteria in solid tumors (RECIST) guidelines [16] for anti-tumor activity, and with NCI common toxicity criteria Version 2 for safety. Patients were withdrawn from the study if evidence of tumor progression was observed. The Institutional Ethical Review Committee approved the study.

Statistical analyses

Time to progression was measured as a period from the start of this treatment to the identifiable time for progression. Survival time was measured from the start of the present treatment until death or last follow-up. The Kaplan–Meier method was used to calculate survival curves. Survival differences between subgroups were compared using the log-rank test. The chi-square test was used to compare the percentage of patients in each group.

Primary endpoints were response rate and toxicity; secondary endpoints were survival and time to pro-

gression. We chose a 50% response rate as a desirable target level and a 25% response rate as an undesirable target. Our design had a power in excess of 95% and less than 20% type I error, requiring 26 patients. Considering the percentage of probable dropout cases, 29 patients were required.

Results

Patient characteristics

Twenty-nine patients were enrolled in this study from March 2000 to June 2002. All patients were assessed for toxicity, response and survival. Characteristics of the 29 patients are listed in Table 1. There were 11 refractory cases and 18 sensitive cases against the first-line chemotherapy.

Efficacy of treatment

The mean number of cycles administered per patient was three, and ranged from one to seven. There were no cycles of dose reduction. One patient achieved a complete response (CR) and 19 patients showed partial response (PR). Overall response rate was 69% (20/29) [95% confidence interval (CI) 52–86%]. The response rate was 83% (15/18, 95% CI: 66–100%) in sensitive cases and 45% (5/11, 95% CI: 16–75%) in refractory cases, with significant differences between the two groups ($P < 0.01$). The median time to progressive disease was 16.4 weeks [21.7 weeks in sensitive pts and 15.3 weeks in refractory pts ($P = 0.32$)]. The overall median survival time was 29.6 weeks (Fig. 1) with no significant differences between sensitive cases (34.1 weeks) and refractory cases (23.1 weeks) ($P = 0.085$). The median survival time differed significantly between PS 0 or 1 patients (46.9 weeks) and PS 2 patients (16.3 weeks) ($P < 0.001$). The 1-year survival rate was 38% (11/29).

Toxicities

Table 2 lists the toxicities observed during this study. Hematological and blood biochemical reactions included a high incidence of leukopenia and neutropenia, leukopenia, and neutropenia of grade 3 or higher occurred in 55 and 55%, respectively. All neutropenia patients recovered upon treatment with G-CSF. Anemia and thrombocytopenia of grade 3 or higher occurred in 27 and 3%, respectively. Subjective and objective symptoms observed included grade 3 diarrhea in three patients who all showed improvement after administration of anti-cholinergic drugs, and grade 3 pneumonitis in one, who showed rapid recovery following administration of steroids. Other subjective and objective symptoms observed were of grade 2 or less and included nausea in 34%, vomiting in 10%, alopecia in 59%, neuropathy in 28%, and flushing in 17%. All of these toxicities disappeared or improved by symptomatic treatment. There were no toxic deaths.

Discussion

No standard chemotherapy for recurrent SCLC has been established since only two Phase III clinical studies have been reported to date on chemotherapy for this disease [13, 17]. In contrast, many studies have been undertaken on salvage chemotherapy for recurrent SCLC, with monotherapy with new third-generation anti-cancer agents and platinum-based multi-drug chemotherapy being the mainstay in recent years [1, 4, 5, 8–10, 14, 15]. Some institutions administer anti-cancer drugs on a weekly basis (weekly chemotherapy) [1, 4, 7, 10]. This treatment regimen makes it possible to titrate the dose of anti-cancer drugs depending on adverse reactions and the patients' physical condition after starting the treatment by dividing the dose into some installments.

The results reported with weekly chemotherapy are summarized in Table 3 [1, 4, 7, 10]. While the study by Goto et al. [4] included only sensitive cases, all other studies included 35–64% of refractory cases. The overall response rate ranged between 31% and 88%: 37–91% in sensitive cases and 23–83% in refractory cases. No study, apart from ours, reported any significant difference between sensitive and refractory cases. The overall median survival time was 6.1–11.8 months with no significant differences between sensitive and refractory cases [10]. In our study, the median survival time was 46.9 weeks in PS 0 or 1 patients and 16.3 weeks in PS 2 patients ($P < 0.001$). Naka et al. [10] reported significant differences between PS 0 or 1 patients (6.9 months) and PS 2 patients (3.8 months) [10]. Hemotoxicity was the main adverse reaction in all studies. Thrombocytopenia was milder in our study than in other studies. Diarrhea also showed a high incidence in regimens including CPT-11.

Groen et al. [5] reported therapeutic results similar to ours with carboplatin and paclitaxel therapy: overall response rate of 73.5% and overall median survival time of 31 weeks. They administered carboplatin and paclitaxel at AUC 7 and 175 mg/m^2, respectively at an interval of 3 weeks. These doses were 1.7 and 0.88 times that obtained by us. . The main adverse reaction was hemotoxicity in both studies, but thrombocytopenia was milder in our study. In the study by Groen et al., 22 and 4 of 34 patients received RBC transfusions and platelet transfusions, respectively [5].

In a phase III trial, which compared topotecan versus cyclophosphamide, doxorubicin and vincristine (CAV) in patients with recurrent SCLC [17], the response rate was 24.3 and 18.3%, respectively; time to progression 13.3 and 12.3 weeks; median survival time 25.0 and 24.7 weeks; 1-year survival rate 14.2 and 14.4%. In our study, the response rate was 69%, time to progression 16.4 weeks,

Table 1 Patient characteristics

Eligible patients	29
Gender	
Male	26
Female	3
Age (years)	
Median	63
Range	43–74
Performance status	
0	9
1	10
2	10
Disease extent at relapse	
Limited disease	7
Extensive disease	22
Relapse type	
Refractory case	11
Sensitive relapse case	18
Prior therapy	
Chemotherapy alone	21
Chemotherapy and irradiation	8
Prior chemotherapy regime	
CBDCA + ETOP	3
CDDP + ETOP(PE)	11
CODE + PE	1
CDDP + CPT-11(PI)	9
CDDP + ETOP + CPT-11	3
PE + PI	2
Response to prior chemotherapy	
Complete response	4
Partial response	21
Stable disease	3
Progressive disease	1

CBDCA carboplatin, *ETOP* etoposide, *CDDP* cisplatin, *CODE* cisplatin/vincristine/doxorubicin/etoposide, *CPT-11* irinotecan

median survival time 29.6 weeks, and 1-year survival rate 37%, and our study showed better therapeutic performance in terms of all four parameters although ours was a pilot study and direct comparisons cannot be made.

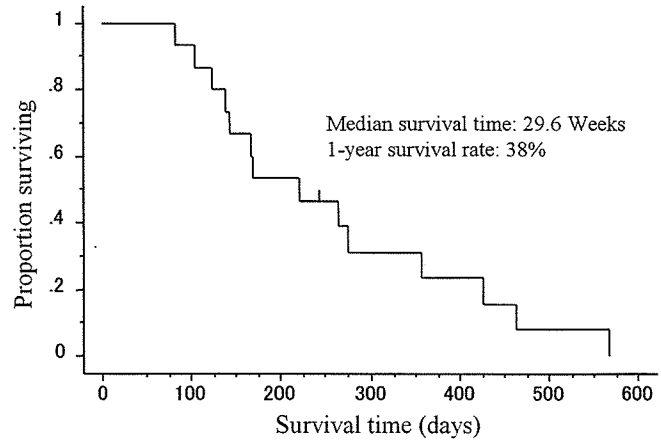

Fig. 1 Kaplan–Meier estimated overall survival curves. Median survival time, 29.6 weeks; 1-year survival rate, 38%

In Japan, cisplatin and irinotecan chemotherapy is the standard therapy for untreated patients in extensive SCLC. Only 8 of 40 patients in the study by Goto et al. [4] and 14 of 29 in our study received irinotecan-based regimens in initial therapy, and no other weekly chemotherapy studies included in Table 3 used such regimens. Carboplatin and paclitaxel combination chemotherapy appears rational in patients with recurrence following initial therapy with cisplatin and irinotecan because the two regimens are not cross resistant.

Conclusion

Weekly chemotherapy with paclitaxel and carboplatin is tolerable and an active regimen for patients with refractory or relapsed SCLC. It is to be recommended as a candidate regimen in planning a phase III clinical study in refractory or relapsed SCLC, and this regimen will ultimately be evaluated in a phase III clinical study.

Table 2 Toxicities ($n = 29$)

	Grade (common toxicity criteria)				Grade ≤ 3 (%)
	1	2	3	4	
Leukopenia	1	7	14	2	16 (55%)
Neutropenia	1	5	9	7	16 (55%)
Anemia	5	8	6	2	8 (27%)
Thrombocytopenia	8	3	1	0	1 (3%)
Diarrhea	7	0	3	0	3 (10%)
Pneumonitis	0	0	1	0	1 (3%)
Nausea	9	1	0	–	
Vomiting	3	0	0	–	
Fatigue	3	3	0	0	
Alopecia	17	0	–	–	
Neuropathy	8	0	0	0	
Flushing	5	–	–	–	
Edema	4	0	0	0	
Arthralgia	3	0	0	0	
Rash	3	0	0	0	
Arrythmia	2	0	0	0	

Table 3 Weekly chemotherapy studies for relapsed small-cell lung cancer

References	Regimen	No. of pts	% of ref pts (%)	RR	RR in sen pts (%)	RR in ref pts (%)	MST (months)
7	CODE	17	35	88	91	83	8.2
10	CPT-11/CBDCA	28	46	31	37	23	6.1
1	CPT-11/CDDP	25	64	80	78	81	7.9
4	CPT-11/CDDP/ETOP	40	0	78	78	–	11.8
Present study	CBDCA/PTX	29	38	69	83	45	7.4

pts patients, *ref* refractory, *sen* sensitive, *RR* response rate, *MST* median survival time, *CODE* cisplatin/vincristine/doxorubicin/etoposide, *CPT-11* irinotecan, *ETOP* etoposide, *CDDP* cisplatin, *PTX* paclitaxel, *CBDCA* carboplatin

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A Phase II Study of Docetaxel and Infusional Cisplatin in Advanced Non-Small-Cell Lung Cancer

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Key Words
Non-small-cell lung cancer · Chemotherapy · Cisplatin · Docetaxel · Infusion, continuous

Abstract
Background: To evaluate the efficacy and safety of combination chemotherapy of cisplatin (5-day continuous infusion) and docetaxel for the treatment of previously untreated patients with advanced non-small-cell lung cancer (NSCLC). ***Materials and Methods:*** Eligible patients had an ECOG performance status of 0–2 with measurable NSCLC. Patients received continuous infusion cisplatin 20 mg/m^2/day on 5 days and bolus docetaxel 60 mg/m^2/day (day 1; PiD therapy) at a 4-week interval. ***Results:*** Forty-three patients were enrolled. The mean number of cycles administered per patient was 2, and ranged from 1 to 4. The response rate was 49% (95% confidence interval, 33.9–63.8%). The median survival time was 47 weeks and the 1-year survival rate was 47%. The major toxic effects were grade 3 or 4, neutropenia (88%), leukopenia (81%), thrombocytopenia (14%) and anemia (42%). There were no treatment-related deaths. ***Conclusion:*** PiD therapy was a well-tolerated and active regimen for patients with advanced NSCLC. The major toxicity was neutropenia.

Introduction

Unresectable non-small-cell lung cancer (NSCLC) is known to have an extremely poor prognosis, and its standard treatment remains to be established. The most common chemotherapy for NSCLC is a combination treatment consisting of 2 or 3 drugs including cisplatin (CDDP) as a key drug. The combination treatments have response rates of 30–50%, and have been proven to prolong survival time in clinical stages III [1] and IV [2, 3]; however, the response is only limited.

In recent years, new anticancer drugs have been developed and used for the treatment of NSCLC. Docetaxel is a new hemisynthetic anticancer agent originating from its precursor, 10-deacytylbaccatin III, extracted from the needle leaves of the European yew tree, *Taxus baccata* L. Docetaxel affects microtubules, and shows its cytotoxicity by prematurely stabilizing mitotic microtubules. In phase II clinical studies for the treatment of NSCLC carried out in Europe and the USA, docetaxel showed a response rate of about 30% in previously untreated patients with a better survival time [4, 5]. A major side effect of docetaxel is dose-dependent edema that is proportional to bone marrow suppression. Since hypersensitivity is particularly limiting, it is worth noting that docetaxel can be given by intravenous infusion in a short period of time without any pretreatment.

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In the Japan phase I study, dose-limiting toxicity of docetaxel was found to be leukopenia (neutropenia), and its recommended dose was set at 60 mg/m^2 [6]. In the multicenter phase II clinical study for the treatment of NSCLC carried out in Japan, a response rate of 19% was shown in untreated patients with predominant toxicities of leukopenia and neutropenia [7].

Currently, cisplatin is the active agent for treating NSCLC, and combination chemotherapy consisting of 2 or 3 drugs based on CDDP is a major strategy [8]. CDDP can be administered by short-term intravenous infusion, a divided dosage method, continuous administration, and other methods [9, 10]. CDDP cytotoxicity is enhanced by prolonged exposure to low doses of this drug in in vitro studies [11, 12]. Belliveau et al. [13] reported that the area under the concentration-time curve (AUC) achieved for non-protein-bound CDDP was twice as high after 5-day continuous infusion than that observed when an equivalent dose of CDDP was given by short-term bolus infusion. These findings suggest that continuous infusion of CDDP might improve the therapeutic efficacy as compared with that resulting from conventional short-term bolus infusion. However, compared with short-term intravenous infusion, 5-day continuous infusion makes inpatient hospitalization for at least 5 days necessary, and the duration of confinement for the purpose of infusion is lengthy and therefore onerous for the patient. The efficacy and safety of a continuous infusion lasting 5 days (24 h a day) were confirmed in our facility and some other facilities [10, 14–16]. In addition, combination chemotherapy of infusional CDDP with vindesine or CPT-11 was found to have high response rates in treating NSCLC [17, 18].

Cisplatin and docetaxel show nonsynergistic and additive effects in vitro, no cross-resistance and have a relatively nonoverlapping toxicity profile [19]. Therefore, the development of docetaxel in combination with cisplatin is warranted. We conducted a phase II study of docetaxel and infusional cisplatin, in patients with previously untreated advanced NSCLC, and evaluated antitumor activity and the safety of this therapy.

Patients and Methods

Patient Selection

All patients with histologically or cytologically confirmed advanced NSCLC were eligible for this phase II trial. The subjects of this study were patients in clinical stage IV or in stage III with unresectable disease or in whom radiotherapy with curative intent is not possible. Patients with unresectable disease or in whom radiotherapy with curative intent is not possible include those with pleural effusion and dissemination, those with intrapulmonary metastasis within the ipsilateral lobe, those in whom the irradiation field exceeds one half of one lung, those with metastasis to the contralateral hilar lymph nodes, and those with reduced lung function. None of the patients had received prior therapy. Other eligibility criteria included an expected survival of 12 weeks, age ≤75 years, Eastern Cooperative Oncology Group performance score of 0–2, measurable lesions, adequate hematological function (WBC ≥4,000/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥10 g/dl), renal function (serum creatinine ≤1.5 mg/dl, creatinine clearance ≥60 ml/min), and hepatic function (total serum bilirubin ≤1.5 mg/dl, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase less than twice the normal range). The ethical committee of the Tochigi Cancer Center approved the protocols. Written informed consent was obtained in every case stating that the patient was aware of the investigational nature of this treatment regimen. Pretreatment evaluation included medical history, physical examination, complete blood count, bone marrow examination, serum biochemical analyses, chest roentgenogram, electrocardiogram, and urinalysis. All patients underwent a radionuclide bone scan, and computerized tomography of the brain, thorax and abdomen. Complete blood count, biochemical tests, serum electrolytes, urinalysis, and chest roentgenograms were obtained weekly during this phase II trial. Tests of measurable disease parameters such as computerized tomography were repeated every 4 weeks. Staging was according to the 4th edition of the UICC TNM classification.

Treatment

All patients were admitted to the Tochigi Cancer Center Hospital during this trial. The anticancer drug regimen consisted of a combined administration of docetaxel plus infusional cisplatin. Docetaxel was supplied, in concentrated form, in a sterile vial that contained 80 mg of the drug in 2 ml of polysorbate 80. Docetaxel (Taxotere; Aventis) 60 mg/m^2 was diluted in 250 ml of 5% glucose, and was infused over a 1-hour period on day 1. Three hours after completion of the docetaxel infusion, 20 mg/m^2 of cisplatin was given daily for 5 days by continuous intravenous infusion. One third of the daily dose was administered every 8 h dissolved in 800 ml of physiological saline [14]. The course was repeated every 4 weeks. Antiemetic drugs used were granisetron (3 mg/body/day, bolus infusion for 5 days), metoclopramide (3 mg/kg/day, continuous infusion for 5 days), methylprednisolone (125 mg bolus infusion every 8 h, days 1–5), diphenhydramine (30 mg orally, days 1–7) and alprazolam (1.2 mg orally, days 1–7) [15, 16]. In the first course, no routine premedication was given for hypersensitivity reactions or fluid retention. The reason for this was that the incidence of these events was low at the dose of docetaxel (60 mg/m^2) administered in the present study [7]. However, if hypersensitivity reactions or fluid retention occurred, premedications such as corticosteroids or antiallergic agents were allowed in the subsequent courses. Recombinant human granulocyte colony-stimulating factor was administered when leukopenia/neutropenia of grade 4 occurred.

Patients were treated with at least two cycles of therapy unless disease progression or unacceptable toxicity was encountered or the patients did not wish to continue. Patients who experienced grade 4 leukopenia or neutropenia that lasted for 3 or more days, or who experienced grade 4 thrombocytopenia or reversible grade 2 neurotoxicity or grade 3 liver dysfunction, received reduced doses of

both docetaxel and cisplatin (75% of the previous dose) for the next cycle. Patients who experienced stomatitis of grade 3 or more or renal dysfunction of grade 2 or more received a reduced dose of cisplatin (75% of the previous dose) for the next cycle. If neurotoxicity of grade 3 or more occurred, treatment was stopped. Subsequent courses of chemotherapy were started after day 28 when the leukocyte count was 4,000/mm^3 or more, the neutrophil count was 2,000/mm^3 or more, the platelet count was 100,000/mm^3 or more, serum creatinine was less than the upper limit of the normal range, creatinine clearance was 60 ml/min or more, GOT and GPT were less than twice the upper limit of the normal range, and neurotoxicity was grade 1 or less. If these variables did not return to adequate levels by the first day of the next course of chemotherapy, treatment was withheld until full recovery. If more than 6 weeks passed from the time of the last treatment before these criteria were satisfied, the patient was taken off the study, but still included in the analysis. In the case of stable or progressive disease after two courses of treatment, subsequent therapy was left to the discretion of the physician in charge of the patient.

Assessment of Response to Treatment and Toxicity

The response to treatment was evaluated with WHO criteria. The criteria for response were as follows. Complete response was defined as the complete disappearance of all evidence of tumor for at least 4 weeks. Partial response was defined as a ≥ 50% reduction in the sum of the product of the two greatest perpendicular diameters of all indicator lesions for at least 4 weeks and no appearance of new lesions or progression of any lesion. Progressive disease was defined as a ≥25% increase in the tumor area or the appearance of new lesions. All other circumstances were classified as no change. Toxicity was graded according to the common toxicity criteria (version 2).

Statistical Analyses

The primary end point was the objective response rate. The duration of each response was defined as the number of days from the documentation of the response until tumor progression. Survival curves from registration until death were generated by the method of Kaplan and Meier. We chose a 40% response rate as a desirable target level, and a 20% response rate as undesirable. The study design had the power to detect a response of greater than 90%, with less than 5% error. Therefore, we needed 23 assessable patients in first stage and 20 in second stage, according to the mini-max design of Simon. We decided to stop the study if fewer than 5 patients responded in the first stage.

Results

Patient Characteristics

Forty-three patients were enrolled in this study from July 1997 to June 1999 and received 105 cycles of the regimen. Table 1 shows the patient characteristics. There were 14 women and 29 men with a median age of 61 years (range 34–75). One patient had stage IIIA, 7 patients stage IIIB, and 35 patients stage IV disease. In stage IIIA, 1 patient classified as c-T3N2M0 had lung cancer with a bulky tumor (10 cm), associated with extranodal and N2 involvement. Among the 7 stage IIIB patients, there were three T4 cases in which pleural effusion and pleural dissemination were present, two T4 cases of intrapulmonary metastasis in the ipsilateral lobe, and two T4N3 cases with mediastinal infiltration and supraclavicular fossa lymph node metastasis.

Table 1. Patient characteristics

Patients	43
Sex (M/F)	29/14
Age[1], years	61 (34–75)
Performance status: 0/1/2	9/30/4
Stage: IIIA/IIIB/IV	1/7/35
Histology: Ad/Sq/Other	27/14/2

Ad = Adenocarcinoma; Sq = squamous cell carcinoma.

[1]Value represents median with the range given in parentheses.

Treatments Administered

The mean number of cycles administered per patient was 2, and ranged from 1 to 4. In 99 of 105 cycles (94%), PiD was administered at 4-week intervals. In 5 of 6 cycles, in which cisplatin could not be administered at a 4-week interval, it was given a week later. As for the remaining cycle, it was administered 6 weeks later. The reason for the delay of the administration was the patient's request for 1 cycle and neutropenia in 5 cycles. Dosage was reduced in 7 cycles (7%). Reductions in dosage of docetaxel and cisplatin were made, respectively, in 6 cycles (6%) and 7 cycles (7%). The former reduction was made because 6 cycles showed neutropenia grade 4, and the latter reduction was made because 5 cycles showed neutropenia grade 4, and 1 cycle showed both neutropenia grade 4 and creatinine grade 3, and 1 cycle showed creatinine grade 2.

Response to Treatment and Survival

The response rate was 49% (95% confidence interval, CI, 33.9–63.8%); a complete response was observed in 1 and partial response in 20 patients (table 2). The median duration of the response was 39.2 weeks (range 5–147 weeks). The median survival time was 47 weeks (95% CI, 6–152 weeks) and the 1-year survival rate was 47% (fig. 1). Two patients are still alive.

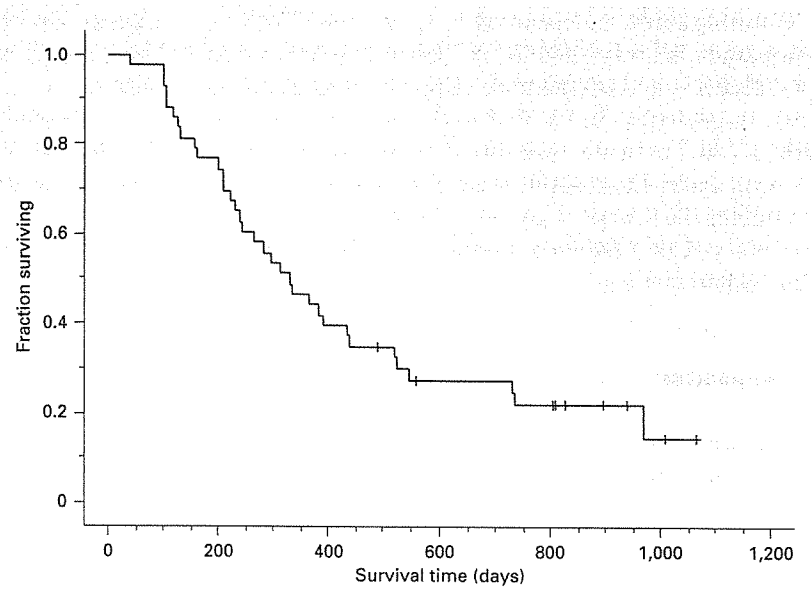

Fig. 1. Kaplan-Meier estimated overall survival curves. Median survival time was 47 weeks; 1-year survival rate was 47%.

Table 2. Chemotherapeutic evaluation (n = 43)

Cycles[1]	2 (1–4)
Response: CR/PR/NC/PD	1/20/20/2
Response rate, %	49
Response duration, weeks	
Average	39.2
Range	5–147
1-year survival rate, %	47

CR = Complete response; PR = partial response; NC = no change; PD = progressive disease.

[1]Value represents average with the range in parentheses.

Table 3. Toxicity (n = 43 patients)

	Maximum toxicity terms of CTC grade					Grade ≥3
	0	1	2	3	4	%
Leukopenia	1	1	6	29	6	81
Neutropenia	1	0	4	13	25	88
Anemia	1	6	18	18	–	42
Thrombocytopenia	25	5	7	6	0	14
Creatinine	23	18	1	1	0	2
SGOT/SGPT	30	12	1	0	0	0
Vomiting	5	7	31	0	–	0
Diarrhea	20	16	7	0	0	0
Alopecia	20	22	1	–	–	
Edema	36	6	1	0	–	0
Neuropathy	40	3	0	0	0	0

Figures represent number of patients. CTC = Common toxicity criteria; SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvic transaminase.

Toxicity

Table 3 shows the types and grades of toxicities resulting from the treatment, using the common toxicity criteria. All 43 patients could be evaluated for toxic reactions. The major toxicity was myelosuppression. Leukopenia <2,000/mm^3 (grade 3 or 4) was observed in 35 patients (81%), of whom 6 patients showed grade 4. Neutropenia <1,000/mm^3 (grade 3 or 4) was observed in 38 patients (88%), of whom 25 patients showed grade 4. Eight patients developed febrile neutropenia. Thrombocytopenia <5 × 10^4/mm^3 (grade 3 or 4) was observed in 6 patients (14%), and a hemoglobin nadir (grade 3) in 18 patients (42%). There were no episodes of bleeding or fluid overload.

Vomiting grade ≥2 occurred in 31 patients (72%). Diarrhea grade ≥2 was observed in 7 patients (16%). Grade 1 or 2 alopecia and edema were observed in 23 and 7 patients, respectively. In the first cycle, creatinine showed grade ≥2 in 2 patients, resulting in transient rises. In the following cycle, the creatinine level was kept at grade 1 by reducing the dosage of cisplatin. Grade 1 or 2 skin rash was observed in 3 patients. Finally, there were no treatment-related deaths.

Discussion

Cisplatin is one of the key drugs for the treatment of NSCLC. Its high response rate of 40% and safety when it was given alone by continuous infusion over 5 days [14] are confirmed.

Docetaxel is also an active agent to treat NSCLC, and docetaxel of 60 mg/m^2/day (day 1), a recommended dose in Japan, showed a response rate of 19% [7]. Docetaxel has no cross-resistance with cisplatin, and in clinical practice, docetaxel was effective in some patients who were resistant to cisplatin [19]. In addition, additive effects are confirmed between cisplatin and docetaxel, and major side effects of the two drugs are different.

This was a phase II study to determine the usefulness and safety of combination chemotherapy of cisplatin (5-day continuous infusion) and docetaxel for the treatment of advanced NSCLC. The response rate in this study was 49%, which is higher than with docetaxel alone. In comparison with other combination therapies, response rates were 39–42% for cisplatin (bolus) and docetaxel [20, 21], and 58.5% for cisplatin (infusion) and irinotecan with G-CSF. In combination with cisplatin (bolus) and newly developed anticancer agents, the response rates were 44% with paclitaxel [22], 31% with gemcitabine [23], and 26% with vinorelbine [24]. Although these studies differed as regards patients' backgrounds, generally, combination therapies showed better response rates than docetaxel alone.

In our study, side effects predominantly involved hematological toxicity (leukopenia, neutropenia, and anemia). Fever associated with neutropenia was observed in 8 (23%) of 43 patients, and they were treated by administering antibiotics. Hematological toxicities were similar to those in other combination therapies [20, 21]. Nonhematological toxicities were mild, with only 1 patient showing an increased creatinine level of grade 3. The increase was transient, and soon returned to normal. Peripheral edema was observed in only 16%, which was markedly lower than the 24–46% found in other studies [5, 25, 26]. When accumulated doses of docetaxel exceeded 500 mg/m^2, the incidence of edema increased, and at a dose of 85 mg/m^2 or less, eruption was not observed [27]. The dosage was 60 mg/m^2 in our study, and no patients received 500 mg/m^2. There were no side effects concerning hypersensitivity or treatment-related deaths.

We carried out a phase II study of combination treatment of cisplatin (5-day continuous infusion) and docetaxel in 43 patients with NSCLC. The response rate was 49%, and median survival time was 47 weeks. A major side effect was neutropenia. A combination treatment of infusional cisplatin and docetaxel is a tolerable and active regimen for patients with advanced NSCLC. It is to be recommended as a candidate regimen in planning a phase III clinical study in advanced NSCLC, and this regimen will ultimately be evaluated in a phase III clinical study.

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Schedule-Dependent Interactions Between Pemetrexed and Cisplatin in Human Carcinoma Cell Lines In Vitro

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The combination of pemetrexed and cisplatin shows good clinical activity against mesothelioma and lung cancer. In order to study the potential cellular basis for this, and provide leads as to how to optimize the combination, we studied the schedule-dependent cytotoxic effects of pemetrexed and cisplatin against four human cancer cell lines in vitro. Tumor cells were incubated with pemetrexed and cisplatin for 24 h at various schedules. The combination effects after 5 days were analyzed by the isobologram method. Both simultaneous exposure to pemetrexed and cisplatin for 24 h and sequential exposure to cisplatin for 24 h followed by pemetrexed for 24 h produced antagonistic effects in human lung cancer A549, breast cancer MCF7, and ovarian cancer PA1 cells and additive effects in colon cancer WiDr cells. Pemetrexed for 24 h followed by cisplatin for 24 h produced synergistic effects in MCF7 cells, additive/synergistic effects in A549 and PA1 cells, and additive effects in WiDr cells. Cell cycle analysis of MCF7 and PA1 cells supported these findings. Our results suggest that the simultaneous clinical administration of pemetrexed and cisplatin may be suboptimal. The optimal schedule of pemetrexed in combination with cisplatin at the cellular level is the sequential administration of pemetrexed followed by cisplatin and this schedule is worthy of clinical investigations.

Key words: Pemetrexed; Cisplatin; Isobologram; Synergism; Antagonism

INTRODUCTION

Pemetrexed (multitargeted antifolate) is a novel antifolate that inhibits multiple points in folate metabolism including thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase (1–3). Preclinical studies of pemetrexed have demonstrated antitumor activity against a variety of human cancer cells in preclinical models (4). The optimal dose and schedule of pemetrexed was considered to be 500 mg/m^2 in a 10-min infusion once every 3 weeks (5,6). Clinical trials of pemetrexed showed a broad activity against a variety of solid tumors including malignant mesothelioma, and colorectal, pancreas, lung, head and neck, gastric, bladder, and breast cancers (6–14). Dose-limiting toxicities included neutropenia, mucositis, diarrhea, and severe nausea and vomiting (5,6). Patients with a folate-deficient state were associated with severe toxicity, and folate and cobalamin administration before pemetrexed has been introduced in clinical trials (9,13).

Combination chemotherapy has become a standard in the treatment of cancer, based upon theoretical advantages and on proven clinical efficacy. The clinical studies of pemetrexed and platinums (e.g., cisplatin, carboplatin, and oxaliplatin) in combinations have been used against malignant mesothelioma and non-small cell lung cancer, and the promising activity of this combination has been observed (15–19). The wide range of antitumor activity of pemetrexed and platinums (20), their different cytotoxic mechanisms and different toxic profiles, and the absence of cross-resistance provide a rationale for using combinations of these agents.

The cytotoxic action of cisplatin is considered to be the result of the formation of cisplatin–DNA adducts

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(20). Pemetrexed treatment may influence adduct formation by cisplatin or the repair of formed adducts, because pemetrexed inhibits both pyrimidine and purine synthesis. The disturbances of the cell cycle produced by pemetrexed and cisplatin may also influence the cytotoxic effects of each other because these agents are cell cycle specific (21,22).

These suggest that the drug schedule may play a significant role in the outcome, and therefore the design of a protocol using them in combination may requires careful consideration. Schedule-dependent interactions have been observed for the combinations of pemetrexed and gemcitabine (23), doxorubicin (24), or paclitaxel (25) in in vitro studies. Because experimental studies for the combination of pemetrexed with cisplatin are limited (26, 27), the optimal schedule of this combination is obscure.

The present study aimed at elucidating the cytotoxic effects of combinations of pemetrexed and cisplatin in various schedules on four human carcinoma cell lines. Our data suggest that the simultaneous administration of pemetrexed and cisplatin may be suboptimal for this combination and the optimal schedule of this combination at the cellular level is the sequential administration of pemetrexed followed by cisplatin.

MATERIALS AND METHODS

Cell Lines

The human lung cancer A549, the breast cancer MCF7, the ovarian cancer PA1, and the colon cancer WiDr cells were used. These cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.) and antibiotics. The doubling times of A549, MCF7, PA1, and WiDr cells in our experimental conditions were 20–24 h.

Drugs

Pemetrexed was kindly provided by Eli Lilly and Company (Indianapolis, IN). Cisplatin was purchased from Nihon Kayaku Co. (Tokyo). Drugs were diluted with RPMI-1640 plus 10% FBS.

Cell Growth Inhibition Using Combined Anticancer Agents

On day 0, cells growing in the exponential phase were harvested with 0.05% trypsin and 0.02% EDTA and resuspended to a final concentration of 5.0×10^3 cells/ml in fresh medium containing 10% FBS and antibiotics. The cell suspensions (100 μl) were dispensed using a multichannel pipette into the individual wells of a 96-well tissue culture plate with a lid (Falcon, Oxnard, CA). Each plate had one 8-well control column containing medium alone and one 8-well control column containing cells without drug. Eight plates were prepared for each drug combination. The cells were preincubated overnight to allow attachment.

Simultaneous Exposure to Pemetrexed and Cisplatin

After 16–20-h incubation for cell attachment, solutions of pemetrexed and cisplatin (50 μl) at different concentrations were added to the individual wells. The plates were also incubated under the same conditions for 24 h. The cells were then washed twice with culture medium containing 1% FBS, and then fresh medium containing 10% FBS (200 μl) and antibiotics was added. The cells were incubated again for 4 days.

Sequential Exposure to Pemetrexed Followed by Cisplatin or Vice Versa

After 16–20-h incubation, medium containing 10% FBS (50 μl) and solutions (50 μl) of pemetrexed (or cisplatin) at different concentrations was added to the individual wells. The plates were then incubated under the same conditions for 24 h. The cells were washed

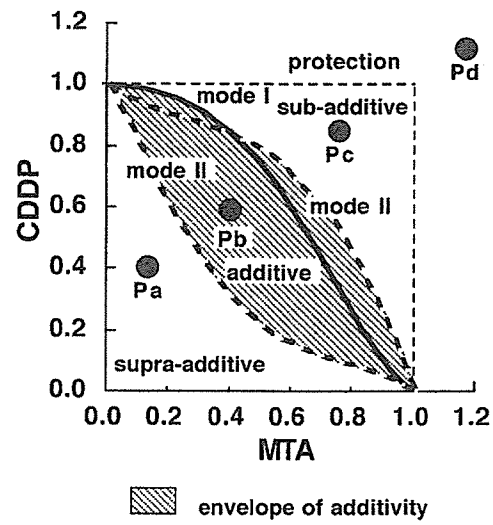

Figure 1. Schematic representation of an isobologram (29). The envelope of additivity, surrounded by mode I (solid line) and mode II (dotted lines) isobologram lines, was constructed from the dose–response curves of pemetrexed (MTA) and cisplatin (CDDP). The concentrations that produced 80% cell growth inhibition were expressed as 1.0 in the ordinate and the abscissa of all isobolograms for MCF7, PA1, and WiDr cells, while the concentrations that produced 50% cell growth inhibition were expressed as 1.0 in the ordinate and the abscissa of all isobolograms for A549 cells. The combined data points Pa, Pb, Pc, and Pd show supra-additive, additive, subadditive, and protective effects, respectively.

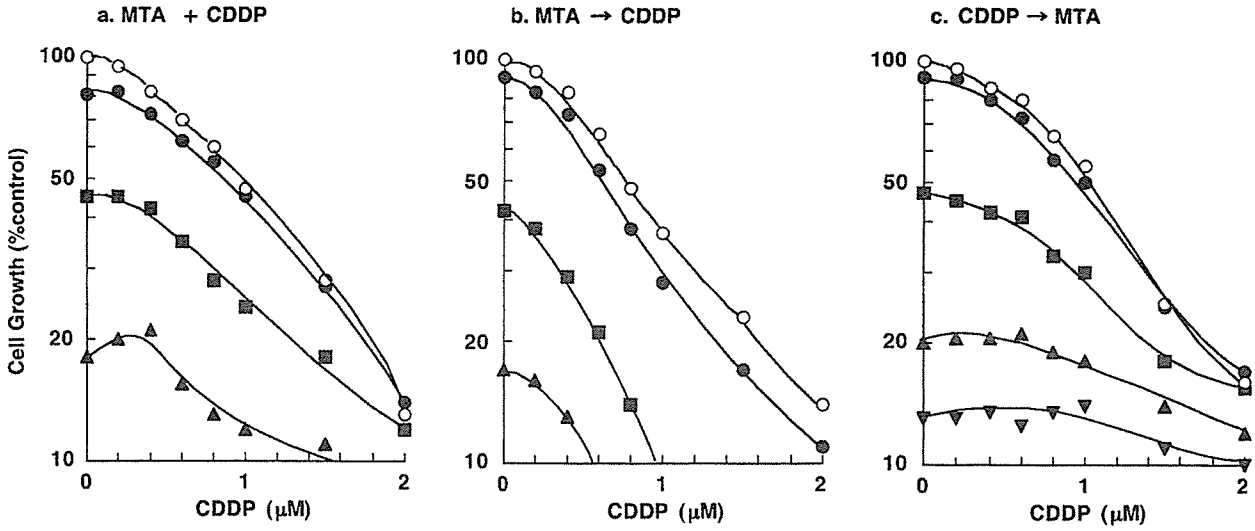

Figure 2. Schedule dependence of the interaction between pemetrexed and cisplatin in PA1 cells. Cells were exposed to these two drugs simultaneously for 24 h (a), pemetrexed first for 24 h followed by cisplatin for 24 h (b), or the reverse sequence (c). The cell number after 5 days was measured using the MTT assay and was plotted as a percentage of the control (cells not exposed to drugs). The concentrations of cisplatin are shown on the abscissa. The concentrations of pemetrexed were 0 (open circles), 20 (filled circles), 50 (filled squares), 100 (filled upward triangles), and 200 (filled downward triangles) nM, respectively. Data are mean values for three independent experiments; SE was <20%.

twice with culture medium containing 1% FBS; fresh medium containing 10% FBS (150 μl) and antibiotics was added, followed by the addition of solutions (50 μl) of cisplatin (or pemetrexed) at different concentrations. The plates were incubated again under the same conditions for 24 h. The cells were then washed twice with culture medium, and fresh medium containing 10% FBS (200 μl) and antibiotics was added. The cells were then incubated again for 3 days.

MTT Assay

The cytotoxicity of pemetrexed alone, cisplatin alone, and their combinations was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (28). For all four cell lines examined, we were able to establish a linear relationship between the MTT assay value and the cell number within the range shown.

Isobologram

The dose–response interactions between pemetrexed and cisplatin for the MCF7, PA1, and WiDr cells were evaluated at the IC_{80} level by the isobologram method of Steel and Peckham (Fig. 1) (29). The IC_{80} was defined as the concentration of drug that produced 80% cell growth inhibition (i.e., an 80% reduction in absorbance). Although the drug interaction at IC_{90} or more would be more important than both IC_{80} and IC_{50} for cancer chemotherapy, it is difficult to get reliable data at IC_{90} or more using MTT assay. A549 was resistant to pemetrexed and the interactions between them were evaluated at the IC_{50} level.

We used the isobologram method of Steel and Peckham because this method can cope with any agents with unclear cytotoxic mechanisms and a variety of dose–response curves of anticancer agents. The concept and analysis of the isobologram has been described in detail previously (30,31). The isobologram of Steel and Peckham is very strict for synergism and antagonism.

If the two agents act additively by independent mechanisms, the combined data points would lie near the mode I line (hetero-addition). If the agents act additively by similar mechanisms, the combined data points would lie near the mode II lines (iso-addition). When the data points of the drug combination fell within the area surrounded by mode I and /or mode II lines (i.e., within the envelope of additivity), the combination was described as additive.

A combination that gives data points to the left of the envelope of additivity (i.e., the combined effect is caused by lower doses of the two agents than is predicted) can confidently be described as supra-additive (synergism). A combination that gives data points to the right of the envelope of additivity, but within the square or on the line of the square, can be described as subadditive (i.e., the combination is superior or equal to a single agent but is less than additive). A combination that gives

data points outside the square can be described as protective (i.e., the combination is inferior in cytotoxic action to a single agent). A combination with both subadditive and/or protective interactions can confidently be described as antagonistic.

Data Analysis

The findings were analyzed as described previously (32). When the observed data points from combinations fell mainly in the area of supra-additivity or in the areas of subadditivity and protection, the mean value of the observed data was smaller than that of the predicted minimum data or larger than that of the predicted maximum data, the combinations were considered to have a synergistic or an antagonistic effect, respectively. To determine whether the condition of synergism (or antagonism) truly existed, a Wilcoxon signed-rank test was performed to compare the observed data with the predicted minimum (or maximum) data for an additive effect. Probability values of $p < 0.05$ were considered significant. Because the isobologram of Steel and Peckham is very strict for synergism and antagonism, combinations with $p \geq 0.05$ were defined as having an additive/synergistic (or additive/antagonistic) effect. All statistical analyses were performed using the Stat View 4.01 software program (Abacus Concepts, Berkeley, CA).

Flow Cytometric Analysis

PA1 cells were treated with 0.2 μM pemetrexed alone or 0.5 μM cisplatin alone or their combination simultaneously for 24 h. MCF7 cells were treated with 0.5 μM pemetrexed alone or 5 μM cisplatin alone or their combination simultaneously for 24 h. The cells were also treated with pemetrexed for 24 h followed by cisplatin for 24 h or the reverse sequence. The cells were harvested at 48 h and the cell cycle profiles were analyzed by staining the intracellular DNA with propidium iodide in preparation for flow cytometry with the FACScan CellFIT system (Becton-Dickinson, San Jose, CA). A DNA histogram was obtained by analyzing 25,000 cells with the ModFIT program (Becton-Dickinson) (33).

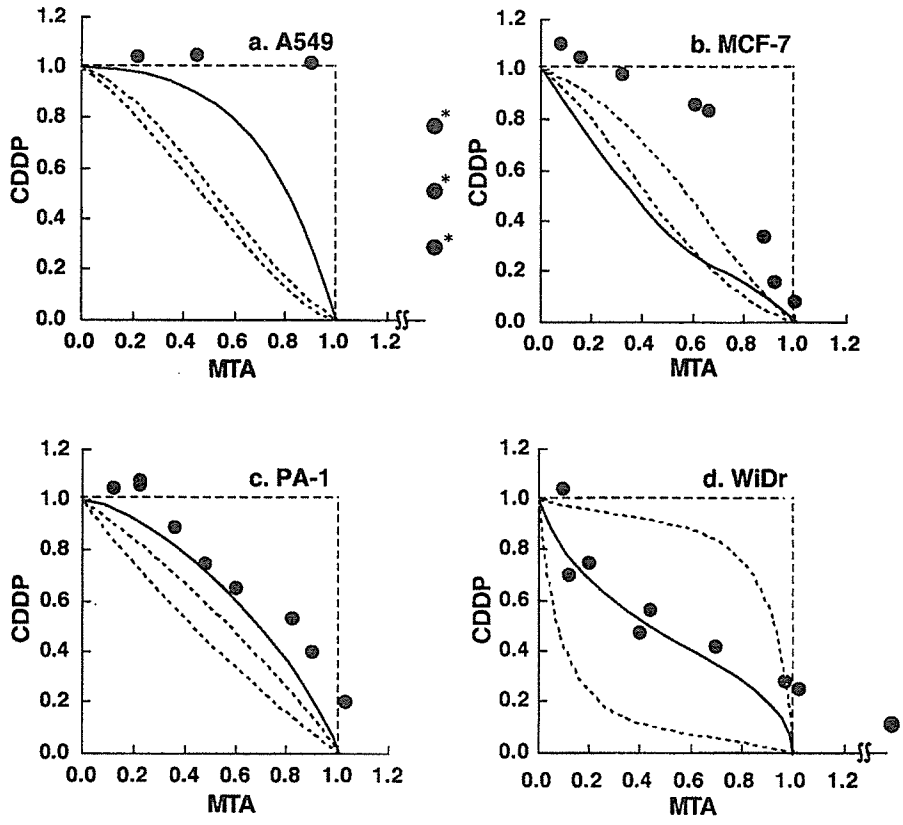

Figure 3. Isobolograms of simultaneous exposure to pemetrexed and cisplatin for 24 h in A549 (a), MCF7 (b), PA1 (c), and WiDr (d) cells. For the A549, MCF7, and PA1 cells, the combined data points fell in the areas of subadditivity and protection. For the WiDr cells, the combined data points fell mainly within the envelope of additivity. Data are mean values for at least three independent experiments; SE was <25% (*except the data).

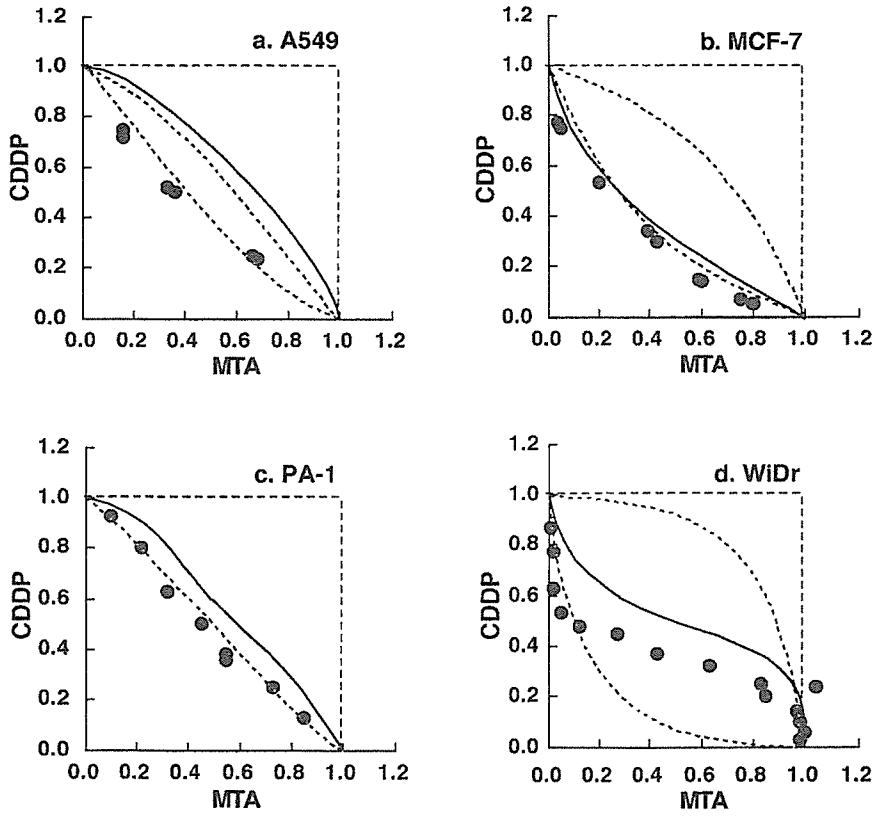

Figure 4. Isobolograms of sequential exposure to pemetrexed (24 h) followed by cisplatin (24 h) in A549 (a), MCF7 (b), PA1 (c), and WiDr (d) cells. For the A549, MCF7, and PA1 cells, all or most of the data points of the combinations fell within the envelope of additivity and in the area of supra-additivity. For the WiDr cells, most of the data points fell within the envelope of additivity. Data are mean values for at least three independent experiments; SE was <20%.

RESULTS

The IC_{80} values of 24-h exposure to pemetrexed for A549, MCF7, PA1, and WiDr cells were >5, 2.5 ± 0.4, 0.10 ± 0.03, and 0.55 ± 0.2 μM, respectively. Because A549 cells were resistant to pemetrexed and the IC_{80} level was not obtained, the interactions between pemetrexed and cisplatin were evaluated at the IC_{50} level. The IC_{50} value of 24-h exposure to pemetrexed for A549 cells was 2.7 ± 0.3 μM.

Figure 2 shows the dose–response curves obtained from simultaneous exposure and sequential exposure to pemetrexed and cisplatin for the PA1 cells. The dose–response curves were plotted on a semilog scale as a percentage of the control, the cell number of which was obtained from the samples not exposed to the drugs administered simultaneously. Dose–response curves in which the pemetrexed concentrations are shown on the abscissa could be made based on the same data (figure not shown). Based upon the dose–response curves of pemetrexed alone and cisplatin alone, three isoeffect curves (mode I and mode II lines) were constructed. Isobolograms at the IC_{80} or IC_{50} levels were generated based upon these dose–response curves for the combinations.

Simultaneous Exposure to Pemetrexed and Cisplatin

Figure 3 shows isobolograms of the A549, MCF7, PA1, and WiDr cells after simultaneous exposure to pemetrexed and cisplatin for 24 h. For the A549, MCF7, and PA1 cells, the combined data points fell in the areas of subadditivity and protection, respectively. The mean values of the observed data (>1.15, 0.95, and 0.69) were larger than those of the predicted maximum values (0.75, 0.72, and 0.56). The observed data and the predicted maximum data were compared by the Wilcoxon signed-rank test. The differences were significant ($p < 0.05$, $p < 0.02$, and $p < 0.01$), indicating antagonistic effects (Table 1). For the WiDr cells, the combined data points fell mainly within the envelope of additivity. The mean values of the observed data (0.66) were larger than those of the predicted minimum values (0.27), and smaller than those of the predicted maximum values (0.73), indicating additive effects.

Sequential Exposure to Pemetrexed Followed by Cisplatin

Figure 4 shows isobolograms of the A549, MCF7, PA1, and WiDr cells exposed first to pemetrexed for 24 h and then cisplatin for 24 h. For the MCF7 cells, combined data points fell in the area of supra-additivity. The mean values of the observed data (0.40) were smaller than those of the predicted minimum values (0.44) (Table 1). The difference between them was significant ($p < 0.01$), indicating synergistic effects. For the A549 and PA1 cells, combined data points fell in the area of supra-additivity and within the envelope of additivity. The mean values of the observed data were smaller than those of the predicted minimum values (Table 1), but the differences were not significant ($p > 0.05$ and $p > 0.05$), indicating additive/synergistic effects. For the WiDr cells, the combined data points fell within the envelope of additivity and in the areas of supra-additivity and protection. The mean value of the observed data was smaller than the predicted maximum values and larger than that of the predicted minimum values (Table 1), indicating additive effects.

Sequential Exposure to Cisplatin Followed by Pemetrexed

Figure 5 shows isobolograms of the four cell lines exposed first to cisplatin for 24 h and then pemetrexed for 24 h. For the A549, MCF7, and PA1 cells, all or most of the combined data points fell in the areas of subadditivity and protection. The mean values of the observed data were larger than those of the predicted maximum values (Table 1). The differences were significant ($p < 0.05$, $p < 0.02$, and $p < 0.02$, respectively), indicating antagonistic effects. For the WiDr cells, most of the combined data points fell within the envelope of additivity, indicating an additive effect of this schedule.

Flow Cytometric Analysis

Finally, we evaluated the cytotoxic effects of pemetrexed and cisplatin on cancer cells using flow cytome-

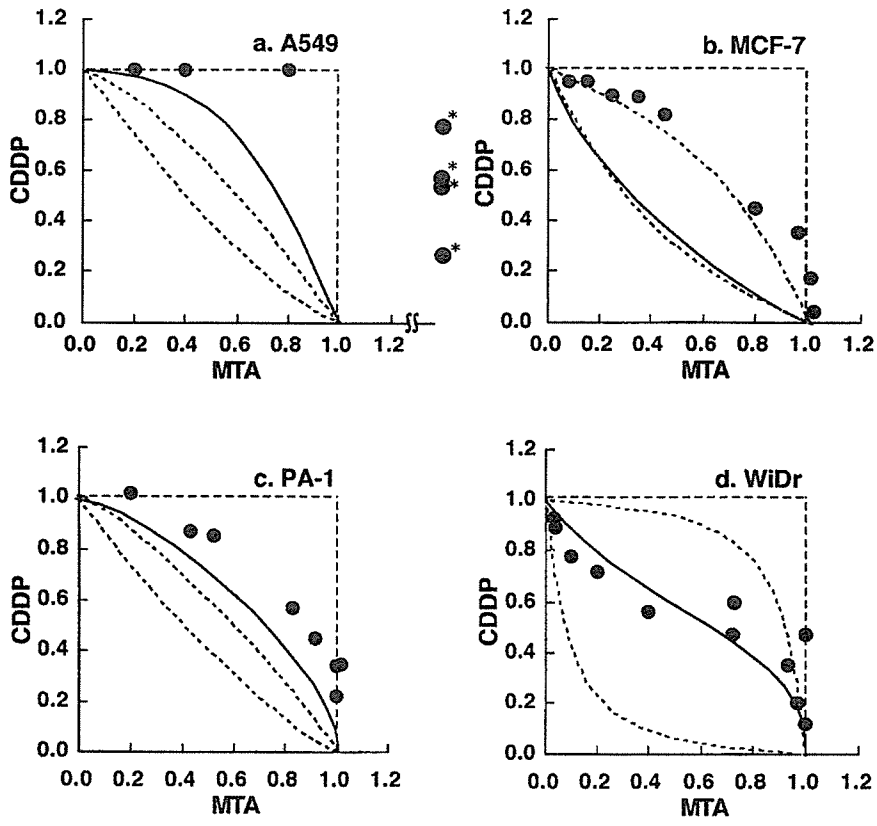

Figure 5. Isobolograms of sequential exposure to cisplatin (24 h) followed by pemetrexed (24 h) in A549 (a), MCF7 (b), PA1 (c), and WiDr (d) cells. For the A549, MCF7, and PA1 cells, all or most of the data points of the combinations fell in the areas of subadditivity and protection. For the WiDr cells, most of the data points of the combinations fell within the envelope of additivity and in the area of subadditivity. Data are mean values for at least three independent experiments; SE was <20%(*except the data).

Table 1. Mean Values of Observed, Predicted Minimum, and Predicted Maximum Data of Pemetrexed (MTA) in Combination With Cisplatin (CDDP) at IC_{80} for MCF7, PA1, and WiDr Cells and at IC_{50} for A549 Cells

Schedule	Cell Line	n	Observed Data	Predictaed Data for an Additive Effect		Effect
				Minimum	Maximum	
MTA + CDDP	A549	6	1.15	0.44	0.75	antagonism ($p < 0.05$)
	MCF7	8	0.95	0.57	0.72	antagonism ($p < 0.02$)
	PA1	9	0.69	0.40	0.56	antagonism ($p < 0.01$)
	WiDr	9	0.66	0.27	0.73	additive
MTA → CDDP	A549+	6	0.45	0.47	0.72	additive/synergism ($p > 0.05$)
	MCF7	9	0.40	0.44	0.78	synergism ($p < 0.01$)
	PA1	8	0.52	0.55	0.64	additive/synergism($p > 0.05$)
	WiDr	15	0.64	0.46	0.84	additive
CDDP → MTA	A549	7	1.14	0.41	0.74	antagonism ($p < 0.05$)
	MCF7	9	0.82	0.52	0.73	antagonism ($p < 0.02$)
	PA1	8	0.75	0.41	0.63	antagonism ($p < 0.02$)
	WiDr	11	0.71	0.21	0.82	additive

try. Cell cycle analysis revealed that pemetrexed and cisplatin arrested PA1 cells in late G_1 to early S phase and G_2/M phase, respectively (Fig. 6A, Table 2). When PA1 cells were exposed to both drugs simultaneously, the cell cycle profile was almost identical to that of a single treatment with pemetrexed, suggesting that the cell cycle effect of pemetrexed is dominant over that of cisplatin. As a result, the apoptosis-inducing effect of cisplatin, which was estimated by an increase in the size of sub-G_1 fraction, was almost completely cancelled in the presence of pemetrexed (Fig. 6A, MTA + CDDP). When PA1 cells were treated with cisplatin first and followed by pemetrexed, the cell cycle pattern closely resembled that of cells treated with cisplatin alone except for a modest increase in G_1 and S phases (Fig. 6A, Table 2, CDDP to MTA). The induction of apoptotsis was less prominent in the CDDP to MTA treatment than in the CDDP treatment (Table 2). In contrast, both apoptosis and G_2/M arrest were enhanced when PA1 cells were treated with pemetrexed first and followed by cisplatin compared with the treatment with either pemetrexed or cisplatin alone (Fig. 6A, Table 2, MTA to CDDP).

We carried out the same analysis with another cancer cell line MCF7 and obtained highly reproducible results. Upon simultaneous addition, the cell cycle effect of cisplatin was almost completely abrogated and the percentage of apoptotic cells was less than that of a single treatment with pemetrexed (Fig. 6B, MTA + CDDP). Similarly, apoptosis was suppressed when MCF7 cells were treated with cisplatin first and followed by pemetrexed compared with the treatment with either pemetrexed or cisplatin alone (Fig. 6B, Table 2, CDDP to MTA). In contrast, the apoptosis-inducing effect of pemetrexed was enhanced by the sequential exposure to cisplatin after pemetrexed (Fig. 6B, Table 2, MTA to CDDP). Overall, these data are fully consistent with the results of isobologram analysis, and provide the molecular basis of the interaction between the two drugs.

DISCUSSION

We found that the cytotoxic interaction between pemetrexed and cisplatin was schedule dependent. Simultaneous exposure to pemetrexed and cisplatin and sequential exposure to cisplatin followed by pemetrexed showed antagonistic effects in A549, MCF7, and PA1 cells, while sequential exposure to pemetrexed followed by cisplatin had a tendency to produce synergistic effects. In the latter schedule, observed data points in A549, MCF7, and PA1 cells were smaller than predicted minimum values for an additive effect (Table 1). WiDr cells showed additive effects in all schedules. The cause of difference in combined effects among cell lines is unknown. The difference may reflect the folate metabolism and the variety of target numbers (enzymes) in the cells. In addition, the isobologram of Steel and Peckham is stricter for synergism and antagonism than other methods. This may also influence the results.

In general, it is difficult to clarify the mechanisms underlying the drug combination. In this study, however, cell cycle analysis provided a clue to understand the molecular basis of schedule-dependent synergism and antagonism of the combination of pemetrexed and cisplatin. The exposure of PA1 and MCF7 cells to pemetrexed for 24 h led to a synchronization of most cells in late G_1 to